Annex 1

U.S. Serial No. 09/308,725

Inventive Step

The Concepts

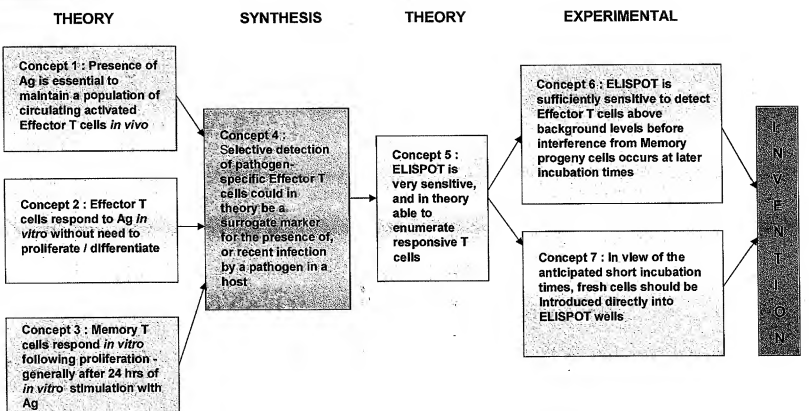

Exhibit A

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41. T. Yoshimoto and W. E. Paul, *J. Exp. Med.* 179, 1285 (1994); T. Yoshimoto, A. Bendelac, C. Watson, J. Hu-Li, W. E. Paul, *Science* 270, 1845 (1995).
42. H. Kosek *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 87, 5248 (1990); O. Lantz and A. Bendelac, *J. Exp. Med.* 180, 1097 (1994); P. Dellabona, E. Padovan, G. Casorati, M. Brockhaus, A. Lanzavecchia, *ibid.*, p. 1171.
43. A. Bendelac *et al.*, *Science* 268, 863 (1995).
44. A. R. Castaño *et al.*, *ibid.* 269, 223 (1995).
45. L. R. Gooding, *Cell* 71, 5 (1992).
46. J. A. Symons, A. Alcami, G. L. Smith, *ibid.* 81, 551 (1995).
47. S. M. Vidal, D. Malo, K. Vogan, E. Skamene, P. Gros, *ibid.* 73, 469 (1993); C. H. Barton, J. K. White, T. I. Roach, J. M. Blackwell, *J. Exp. Med.* 179, 1683 (1994); S. Vida *et al.*, *ibid.* 182, 655 (1995); C. Nathan, *Cell* 82, 873 (1995).
48. J. M. Penninger *et al.*, *Nature* 375, 241 (1995).
49. R. E. Dorig, A. Marcil, A. Chopra, C. D. Richardson, *Cell* 75, 295 (1993).
50. G. J. Kotwal and B. Moss, *Nature* 335, 176 (1988).
51. N. R. Cooper, *Immunol. Today* 12, 327 (1991); M. Partizada, R. Amon, P. J. Lachmann, Z. Fishelson, *J. Exp. Med.* 179, 1625 (1994).
52. J. A. Schifferli and D. K. Peters, *Lancet* II, 957 (1983); K. A. Davies, J. A. Schifferli, M. J. Walport, *Springer Semin. Immunopathol.* 15, 397 (1994).
53. L. C. C. Afonso *et al.*, *Science* 263, 235 (1994); E. Mougneau *et al.*, *ibid.* 268, 583 (1995).
54. T. A. Wynn *et al.*, *Nature* 376, 594 (1995); S. H. Gavett *et al.*, *J. Exp. Med.* 182, 1527 (1995).
55. Y. A. W. Skeiky *et al.*, *J. Exp. Med.* 181, 1527 (1995).
56. V. K. Kuchroo *et al.*, *Cell* 80, 707 (1995).
57. J. Rhoades *et al.*, *Nature* 377, 71 (1995).
58. F. Powrie, R. Correa-Oliveira, S. Mauze, R. L. Coffman, *J. Exp. Med.* 179, 589 (1994); J. D. Katz, C. Benoist, D. Mathis, *Science* 268, 1185 (1995).
59. T. Sumida *et al.*, *J. Exp. Med.* 182, 1163 (1995).
60. Supported by the Wellcome Trust (D.T.F.), National Institutes of Health (grant AI29918 and AI30663) (R.M.L.), and the Burroughs Wellcome Fund (R.M.L.).

Immunological Memory and Protective Immunity: Understanding Their Relation

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The immune system can remember, sometimes for a lifetime, the identity of a pathogen. Understanding how this is accomplished has fascinated immunologists and microbiologists for many years, but there is still considerable debate regarding the mechanisms by which long-term immunity is maintained. Some of the controversy stems from a failure to distinguish between effector and memory cells and to define their roles in conferring protection against disease. Here the current understanding of the cellular basis of immune memory is reviewed and the relative contributions made to protective immunity by memory and effector T and B cells are examined.

An 18th-century natural experiment that occurred on the remote Faroe Islands provided rare insight into the mechanism of protective immunity against infectious diseases. This experiment began in 1781 with a measles outbreak. During the ensuing 65 years, the Faroes remained measles-free until a major outbreak in 1846 that affected 75 to 95% of the population. Ludwig Panum, a Danish physician, investigated this epidemic and made the interesting observation that "of the many aged people still living on the Faroes who had had measles in 1781, not one was attacked a second time" (*1*). This natural experiment had also provided Panum with a control group, and he astutely noted that "all the old people who had not gone through with measles in earlier life were attacked when they were exposed to infection." Panum's classic study made two points: first, that immunity to measles was long-lived; and second, that reexposure to the virus was not essential for maintaining long-term protective immunity. The first point was important but perhaps not that surprising; after all, the Greek historian Thucydides, describing the plague of Athens in 430 B.C., had noted that "the same man was never attacked twice" (*2*). It was Panum's second point, that protective immunity could be sustained in the absence of reexposure to measles virus, that provided a critical insight into the nature of immunological memory. This observation, which was supported by observations made during epidemics of yellow fever in Virginia (*3*) and polio among Eskimo villages in Alaska (*4*), showed that the immune system could remember an encounter that occurred many years ago and that there are inherent (endogenous) mechanisms for sustaining this long-term memory.

The subject of immune memory has been extensively studied, but there is still considerable debate regarding the mechanisms by which protective immunity is maintained (*5*). In this article, we will review our current understanding of the cellular basis of immunological memory and then examine the relative contributions of memory and effector T and B cells to protective immunity. We will also consider differences between protection against mucosal versus systemic infections—a critical issue that is often ignored. We hope this review will provide a framework for trying to understand why protective immunity is long-lived in some situations and of shorter duration in others.

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T Cell Memory

It is well established that T cell memory, as assessed by accelerated recall responses in vivo, is long-lived (*5*). However, the nature of T cell memory has remained controversial, with debate centered around two opposing views (*5*). One view postulates that memory is due to long-lived memory cells that do not require contact with specific antigen for their survival. The other envisions long-term memory as the result of continuous stimulation of T cells by persisting antigen. Although these are interesting and useful hypotheses, they have imposed constraints on the understanding of the cellular basis of T cell memory. For instance, it is conceivable that memory T cells may be antigen-independent but still cycle because of some nonspecific stimulus. It is also plausible that memory cells are long-lived but need antigen stimulation to maintain a state of readiness. Instead of trying to distinguish between the two opposing hypotheses, we will examine memory within the broad framework of the following questions: What is the cellular basis for accelerated recall responses? Are memory T cells distinct from effector cells? What is the lineage of memory T cells? Is specific antigen necessary for the maintenance of T cell memory? What is the life-span of memory T cells? Do these cells cycle? If so, what is the stimulus? Before addressing these questions, it is useful to review the kinetics of primary T cell responses.

Both CD4 and CD8 T cell responses can be broken down into three distinct phases: (i) activation and expansion, (ii) death, and (iii) stability or memory. During the initial phase, which typically lasts about a week, antigen-driven expansion of the specific T cells and their differentiation into effector cells occur. In several viral systems, between 100- and 5000-fold expansion of virus-specific $CD8^+$ T cells takes place (*6–13*). Substantial expansion of $CD4^+$ T cells has also been reported for several antigenic systems (*14*, *15*) and a recent study (*16*) has documented up to 1200-fold expansion of $CD4^+$ T cells responding to pigeon cytochrome c (PCC). A period of death then ensues (between days 7 and 30), during

which most of the activated T cells undergo apoptosis and effector activity subsides as the amount of antigen declines (*6–13, 16, 17*). This contraction of the T cell response is as dramatic as the expansion, and in most instances ≥95% of the antigen-specific T cells disappear. This phenomenon, termed activation-induced cell death (AICD), serves as a mechanism for regulating cell numbers and maintaining homeostasis (*7, 17*). Apoptosis due to Fas (CD95)–FasL interactions has been implicated in AICD (*17*), and a recent study has also documented a role for tumor necrosis factor (TNF) in the apoptosis of activated $CD8^+$ T cells (*18*). The third phase of the T cell response is characterized by a stable pool of memory cells that can persist for many years. The eventual magnitude and duration of T cell immunity are the sum effect of changes occurring in all three phases of the T cell response (Fig. 1).

Accelerated T cell responses seen upon reexposure to antigen are due to increases in the frequency of antigen-specific T cells (a 5- to 100-fold increase, depending on the system) and also to qualitative changes in memory T cells that allow them to respond faster and develop into effector cells more efficiently than do naïve T cells (*5*). Because memory cells express larger amounts of adhesion molecules, they are likely to adhere to antigen-presenting cells (APCs) more efficiently. This may explain why memory cells are activated more readily than are naïve cells and also are able to respond to lower doses of antigen as compared with naïve cells (*5, 14*). This increased responsiveness might also be due to higher affinity interleukin-2 receptors or to changes in the affinity of the T cell receptor (TCR). Somatic hypermutation of TCR V_α and V_β genes is not generally thought to occur in T cells (*16, 19*), but a recent study has shown (*20*) that T cells in germinal centers (GCs) may mutate, albeit at a rate 10 times less than that of B cells. The affinity of T cell responses may also increase by means of clonal selection: Analysis of the $V_\alpha 11/V_\beta 3$ sequences that prevail in the response to PCC in H-2^k mice demonstrates a progressive maturation until particular TCR sequences dominate the secondary response (*16*). Thus, a clonal selection for complementarity-determining region 3 length and ultimately for characteristic amino acids seems likely to drive an increase in the affinity of the receptor for the peptide–major histocompatibility complex (MHC) complex. However, it is not clear if this is a general phenomenon, because a recent study (*21*) found no differences in the TCR repertoire of antigen-specific $CD8^+$ T cells during primary and secondary responses to an MHC class I antigen.

A critical question about T cell memory is whether there is such a thing as a true memory cell. Cell surface markers that distinguish memory T cells from naïve T cells are not useful in differentiating between memory and effector cells (*5*). Therefore, immunologists have relied on size (effector cells are usually large and memory cells small) and functional differences to identify these two populations. By definition, an effector $CD4^+$ T cell actively secretes cytokines, whereas a memory $CD4^+$ T cell has to be stimulated with antigen to start producing large amounts of cytokines. Similarly, effector $CD8^+$ cytotoxic T lymphocytes (CTLs) have direct ex vivo cytotoxic activity, whereas memory CTLs need restimulation with antigen to acquire cytotoxic function. Initial evidence for the existence of distinct populations of effector and memory cells came from work on polyclonal CTL responses to lymphocytic choriomeningitis virus (LCMV) (*22*) and alloantigens (*23*), and more compelling evidence has come from recent studies with monospecific TCR-transgenic CD4 and CD8 cells, in which it is possible to directly visualize the antigen-specific T cells (*14, 24, 25*). However, biochemical markers to identify these two populations are still needed. The presence of perforin granules or increased FasL expression (or both) might serve as useful markers for effector CTLs, because CTLs kill their targets either by the perforin-granzymes secretory pathway or by Fas-FasL interactions (*26*). Other potential markers are genes controlling apoptosis (for example, the Bcl-2 family of proteins, the TNF receptor, and Fas), because effector T cells are more prone to AICD than are memory cells (*5, 27, 28*).

The precise relation (lineage) between memory and effector T cells is not well understood. We do not know if memory and effector cells differentiate along separate pathways as B cells do (model 1, Fig. 2) or if effectors can "rest" as memory cells (model 3, Fig. 2). We are also ignorant of the precise conditions and signals that lead to development of memory versus effector cells. One possibility is that large amounts of antigen plus costimulation in the presence of an inflammatory "milieu" may favor differentiation to effector T cells, whereas antigen plus costimulation in the absence of inflammatory signals or cytokines may result in generation of memory T cells (model 1, Fig. 2). According to this model, T cells activated in the early stages of an infection would receive all the necessary signals for terminal differentiation into effectors, whereas cells that first encounter antigen during the later stages (as antigen and infection are disappearing) would develop into memory T cells. The more conventional model is the linear differentiation pathway (model 3, Fig. 2), in which memory cells are derived directly from effector cells. Adoptive transfer experiments (*24, 25*) with TCR-transgenic cells support this model, but it is not clear if all the transferred cells in these experiments were indeed effector cells. The linear differentiation model has to incorporate a mechanism for discriminating between effectors that die and those that survive. Currently, little is known about this selection process, but TCR affinity may play a role, with higher affinity effector cells surviving as memory cells (*16*). A currently popular model of T cell differentiation proposes that the balance between effector cells and memory cells is governed by the level of stimulation (model 4, Fig. 2). Cells become more and more terminally differentiated with each successive stimulus and cell division (*29*). This is accompanied by an increasing susceptibility to apoptosis. This presumably explains the phenomenon of

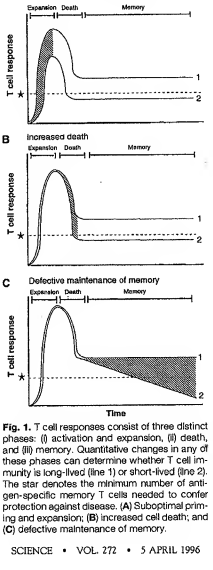

Fig. 1. T cell responses consist of three distinct phases: (i) activation and expansion, (ii) death, and (iii) memory. Quantitative changes in any of these phases can determine whether T cell immunity is long-lived (line 1) or short-lived (line 2). The star denotes the minimum number of antigen-specific memory T cells needed to confer protection against disease. (**A**) Suboptimal priming and expansion; (**B**) increased cell death; and (**C**) defective maintenance of memory.

clonal exhaustion that can occur in TCR-transgenic and virus infection models, in which overwhelming antigen load or activation is a feature (30). Thus, memory T cells can arise only under conditions in which the antigenic load is limited and the stimulation of precursors ceases before a point of no return.

There has been considerable debate regarding the role of specific antigen in maintaining T cell memory (5). At the outset, it should be stated that the presence of antigen is essential to maintenance of effector T cells (although, as noted above, under certain conditions chronic stimulation with large amounts of antigen can result in deletion of effector T cells). It should also be said that periodic reexposure to antigen will enhance the level of T cell memory; experiments showing increases in the number of memory T cells (or level of protective immunity) after reintroduction of antigen should not be taken as evidence that memory is strictly antigen-dependent. The real question is whether the pool of memory T cells formed after infection or immunization can remain relatively stable in the absence of an endogenous depot of specific antigen or is strictly dependent on stimulation by specific antigen. This chronic stimulus could come from a low-grade infection (in the case of pathogens) or from antigen persisting on follicular dendritic cells (FDCs) or both (5, 31, 32). Resolution of this issue is important for practical reasons (vaccines) and also because it defines how we view immune memory. If continuous antigenic stimulation is essential for maintaining memory, then one might question the very existence of immune memory (5, 25).

The experimental approach commonly used to examine the antigen dependence of T cell memory is to adoptively transfer primed T cells into naïve recipients and follow their survival in the apparent absence of antigen (5). The availability of B cell–deficient mice (33) has provided an additional approach to this question since antigen-antibody complexes on FDCs have been implicated in sustaining T cell memory (5, 31, 32). FDCs can trap antigen in the form of antigen-antibody complexes and retain it on their cell surface for long periods of time. It has been postulated that B cells or other APCs pick up this trapped antigen and present it to T cells (5, 31, 32). This mode of antigen loading favors MHC class II presentation, but there is now increasing evidence that certain macrophages can process and present exogenous antigen on MHC class I molecules (34). Thus, antigen depots on FDCs could potentially play a role in sustaining CD4 and CD8 T cell memory.

Both experimental approaches (that is, adoptive transfers and immunization of B cell–deficient mice) have been used to examine the antigen dependence of CD8 and CD4 T cell memory. More extensive studies have been done on CD8 memory (6, 8, 9, 25, 35–40), and the bulk of the evidence indicates that CD8 memory cells can persist in the absence of specific antigen (8, 9, 25, 36–40). Although initial studies (35) on H-Y specific CTLs suggested a requirement for antigen, more recent studies by the same group (37) have shown that CD8 memory to H-Y does not require B cells or CD4$^+$ T cells. In addition, transfer experiments with H-Y-specific TCR-transgenic cells have also documented persistence of memory cells in the absence of H-Y antigen (25). Compelling evidence for persistence of CD8 memory in the absence of specific antigen was provided by Lau *et al.* (8). In these experiments, LCMV-specific memory CD8 cells purified by fluorescence-activated cell sorting, which were free of any viral genetic material (polymerase chain reaction –negative), were adoptively transferred into uninfected mice and were shown to persist and provide protective immunity for >2 years. Müllbacher (36) and Hou *et al.* (9), studying CTL responses to Sendai virus and

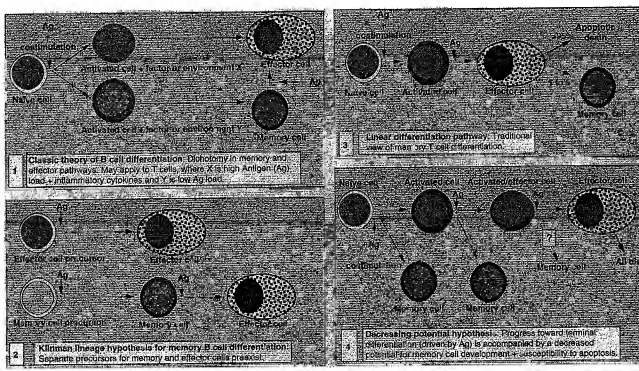

Fig. 2. Models of memory cell differentiation. These are the four basic models we can envisage. They are presented in a simplistic fashion to represent variations on the same theme. For instance, model 1 can represent the B cell paradigm or the theories of T cell differentiation that invoke antigen dose or inflammatory signals as factors that decide the route taken. In all of these models, if the effector cell is depicted as an end cell, then all those cells die within a relatively short time. In all of the models, the memory cells survive for long periods. They can be activated at e later time to develop into effector cells and generate more memory cells.

influenza virus, have come to similar conclusions. Several groups have analyzed CD8 responses in B cell–deficient mice and find that CTL memory to ovalbumin (40), H-Y (37), or LCMV (38) does not wane. Thus, it appears that neither B cells nor antigen-antibody complexes are essential for maintaining $CD8^+$ T cell memory. In addition to these experimental systems, there is also evidence for long-term CTL persistence in humans in the apparent absence of specific antigen. A recent study (39) has shown that vaccinia virus–specific memory CTLs can be detected in individuals vaccinated more than 30 years earlier. It is unlikely that this long-term CTL memory is due to antigen persistence, because vaccinia virus does not cause a chronic or latent infection in humans and there is no possibility of reexposure to vaccinia virus, because vaccination against smallpox virus was stopped in 1977.

Fewer studies have investigated whether antigen is required for the maintenance of $CD4^+$ T cell memory. In one study (35), it was found that in the absence of antigen, T cell help decayed within a few weeks. FDC-maintained antigen was implicated (41) in this decay, and so it is interesting that in B cell–deficient mice the longevity of CD4 T cell memory appears to be compromised (42). However, not all studies of this nature (43) or studies using different approaches (24) have reached this conclusion. Nevertheless, it is tempting to speculate that B cells may be involved in sustaining $CD4^+$ memory (42) and that the rules for maintaining CD8 and CD4 T cell memory are different.

What is the life-span of memory T cells? Turnover studies on cells with a "memory phenotype" in mice (27), sheep (44), and humans (45) have indicated that at least some of these cells divide with regularity. A drawback of such studies is their failure to distinguish between memory and effector cells on the basis of the surface markers used; thus, it is not clear whether the cycling cells are memory cells or effector cells. Studies (46) analyzing the in vivo state of LCMV-specific memory CTLs have shown that a small fraction (5 to 10%) of memory CTL_s are in cycle at a given time. It is not known whether the resting (~90%) and cycling cells represent two distinct populations or whether over the lifetime of the mouse all the memory cells will undergo some small amount of division. Because the total number of memory CTLs remains relatively constant, this amount of turnover must be sufficient to compensate for the rate of cell death. An important question is whether this turnover represents a specific mechanism for maintaining antigen-specific memory or is a general mechanism for maintaining the total number of peripheral T cells. It is well established that, at a population level, peripheral T cells are extremely long-lived (27). For example, thymectomy of adult mice has only a minimal effect on the total number of T cells (47). If there is some cycling of memory T cells, what is the stimulus? There are several possibilities: (i) signaling through the TCR by cross-reactive environmental or self antigens (48, 49), (ii) signaling through adhesion and costimulatory molecules that are up-regulated on memory T cells (5), or (iii) hyperresponsiveness to cytokines because of increased amounts of cytokine receptors (50). These possibilities can be tested experimentally with the use of the appropriate knockout mice and blocking reagents.

Many critical issues of T cell memory (intermitotic life-span, lineage, and so on) still remain unresolved. Adoptive transfer experiments using transgenic T cells bearing single rearrangement, monospecific TCRs should shed light on many of these questions. However, when setting up these experiments, one should be aware that transfer into an empty vessel, such as mice with severe combined immunodeficiency disease, may give rise to exaggerated estimates of life-span as there is no pressure on the reconstituting cells from repopulating precursors from primary lymphoid organs. Conversely, transfer into intact mice may give very short life-span estimates, although there is the potential to achieve a stable chimerism, which would allow study of the subsequent decay of numbers over time (14). The use of TCR-transgenic mice as donors offers us the means to investigate more stringently than ever before the role of antigen dependence in memory cell survival. However, there are pitfalls to avoid; given that cells expand after transfer (24, 51), probably because of encounters with environmental or cross-reactive antigens (48), it seems wise to carry out such experiments with TCR transgenics that are back-crossed onto a recombination activating gene–deficient background, and so avoid nonspecific antigen effects caused by endogenous receptor rearrangements.

B Cell Memory

Secondary or recall antibody responses differ from primary responses in three easily quantifiable ways: (i) they occur more rapidly, (ii) they consist of relatively more immunoglobulin G (IgG), IgA, or IgE than of IgM, and (iii) they are of higher affinity. The accelerated response is the result of increases in the frequencies of antigen-specific B cells and $CD4^+$ helper T cells (memory is a feature of T cell–dependent antibody responses; in general, T cell–independent antigens induce very poor and short-lived memory responses). Differences in the activation requirements of memory B cells and memory T helper cells also enhance the speed of induction of the secondary response, but frequency may be the most important factor: The simple expedient of increasing the frequency of naïve antigen-specific transgenic helper T cells can result in a secondary-type antibody response (52).

The processes of clonal expansion, somatic hypermutation of Ig V regions, affinity selection (53), and much of the isotype switching take place in germinal centers (GCs). Germinal centers are, in fact, specialized sites of memory B cell generation. Inhibition of the GC reaction leads to ablation of secondary antibody responses (54). Advances in our knowledge of memory B cell development have accrued rapidly in recent years and have been reviewed elsewhere (31). Figure 3 depicts some of the important events in memory B cell differentiation.

The question of whether there is a specialized memory or GC precursor cell is one that has aroused controversy. Klinman and colleagues (55) maintain that B cells expressing small amounts of the heat-stable antigen (recognized by J11d antibody) do not take part in primary responses but are the precursors of memory B cells and give rise to GCs, in contrast to $J11d^{hi}$ cells (see model 1, Fig. 2). However, it is clear from sequence analysis that the cells that form proliferating foci in the T zones early in the response (see Fig. 3) can be clonally related to cells in adjacent GCs (56). Whether or not memory B cells develop as a distinct lineage from the cells that generate the primary reponse, it is clear that memory B cells differentiate along a separate pathway from effector or plasma cells (see model 1, Fig. 2). The signals that drive these two pathways are distinct; for instance, CD40L (57) and transcription factors such as B cell–specific activator protein (58) favor memory development, whereas OX40 (59), CD23 (60), and Blimp 1 (61) are important for plasma cell differentiation.

After leaving the GC, memory B cells reenter the pool of lymphocytes that recirculate among secondary lymphoid organs. Some of the recirculating memory B cells soon enter the marginal zone of the spleen and become resident nonrecirculating cells (31). Most B cells that leave the GC appear to express switched Ig isotypes (62). In T-dependent responses, most of the Ig switching may happen in the GC but it is not specific to this site; antigen-specific IgG can be detected before GC formation (31), and IgG antibodies are produced in response to T-independent antigens that elicit no GC reaction. In contrast, somatic hypermutation does seem to be restricted to the GC (56), although not all cells that leave the GC are necessarily mutated (63).

The long-term survival of memory B cells is clearly potentiated by the presence of persistent antigen depots on FDCs (5, 31, 32). The question of whether there is any obligatory requirement for antigen has not been satisfactorily resolved. On the one hand, transfer of primed B cell populations in the absence of antigen led to the disappearance of the memory response within 6 to 12 weeks (64); on the other, BrdU labeling of IgG-expressing antigen-specific B cells 20 weeks after immunization indicated that turnover was low (although turnover of 10% of the memory population during 18 days of BrdU administration is not insignificant) (65). Do memory B cells require T cell help for their survival? One study found that memory B cells could persist for more than 6 weeks in mice depleted of $CD4^+$ T cells (66); however, there was evidence that the anti-CD4 treatment did not abolish all helper function. Additional studies are clearly needed to resolve this issue.

Injection of mice with tetanus toxoid or keyhole limpet hemocyanin (67) can elicit serum antibodies that are detectable during the entire life of a mouse, and people vaccinated with diphtheria or tetanus toxoid can have circulating antibody for more than 25 years (68). Amounts of antibody do decline substantially over this period, but it is remarkable that circulating antibody can still be detected 25 to 50 years after immunization with nonreplicating antigens [the half-life of free antibody molecules is less than 2 weeks (68)]. To obtain such long-lasting antibody responses, three doses must be administered over 1 year; we do not know if this is needed to bring about sufficient memory cell expansion or to deposit sufficient antigen onto FDCs. Conventional wisdom is that in order to maintain the concentration of serum antibody, there must be persistent stimulation to drive a continuing differentiation to plasma cells. This is based on the notion that plasma cells are short-lived, yet evidence supporting this idea is surprisingly scarce. One study has highlighted considerable variation in plasma cell life-span, depending on the isotype produced and the site of production (69). Most of the plasma cells in the spleen and lymph nodes and half of those in the gut lamina propria of mice have a life-span of around 3 days. In contrast, most plasma cells in the bone marrow, the organ that contributes the major proportion of serum antibody in established responses, have a life-span estimated at 3 to 4 weeks (69, 70). If this estimate is correct, the maintenance of serum antibody would still require continued stimulation, but clearly this aspect of long-term antibody responses warrants further investigation, especially in the light of a much older study (71) indicating a very long life-span for a small proportion of plasma cells.

Protective Immunity

The four players involved in protective immunity—plasma cells, memory B cells, effector T cells, and memory T cells—differ in the longevity of their responses, have different maintenance requirements, and act in different ways to confer protection.

Plasma cells provide the first line of defense against infection. The presence of preexisting neutralizing or opsonizing antibody at the site of infection is the most effective mechanism for preventing infection per se and is the key aspect of protective immunity against many viral and most bacterial infections (72). After infection or vaccination, antibody in the serum can persist for decades (68, 72). In contrast, mucosal antibody responses are relatively short-lived (usually a few months to a year or so). This has profound consequences for protective immunity against mucosal infections. It is not coincidental that short-lived immunity is often associated with localized

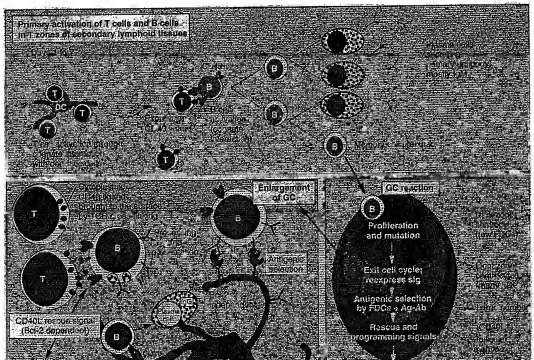

Fig. 3. Development of memory B cells. The primary activation of B cells takes place in T zones of secondary lymphoid organs during the first week after immunization (upper panel). The initial activation of T cells is mediated by dendritic cells. During subsequent T-B interactions, the ligation of CD40 by CD40L on activated $CD4^+$ T cells is crucial for primary antibody production, isotype switching, and progress of B cells into the memory pool. Most B cells activated in T zones differentiate into plasma cells secreting the primary response IgM antibody. Some B cells (memory precursors) enter follicles and begin to proliferate and form GCs. They also start to mutate their Ig receptors; the signals that regulate proliferation and mutation in GCs are unknown. Cells progress through the GC under the influence of programming and rescue signals (lower panels). For memory B cells, there is a two-step selection process, the first involving a screen of antigen-binding capacity and the second involving a cognate interaction to induce CD40L on T cells that can then facilitate long-term memory B cell survival (lower left panel). At certain times, plasma cells arise in the GC after interaction of B cells with CD23 on FDCs. These are mostly IgG-secreting cells and are likely to migrate to the bone marrow.

mucosal infections [for example, rotavirus, respiratory syncitial virus (RSV), rhinoviruses, and so on], whereas long-term protective immunity is a feature of many systemic infections (such as measles, yellow fever, polio, mumps, and smallpox) (*72*). There are exceptions, of course, but the trend is too striking to ignore.

The reasons for the marked differences in the durations of mucosal versus systemic antibody responses are not known. Most of the antibody present in the serum comes from plasma cells residing in the bone marrow (*70*, *73*), whereas mucosal antibody levels mostly reflect the number of plasma cells present at mucosal sites. It is possible that plasma cells have very different life-spans at these two anatomical locations. The bone marrow microenvironment may provide the right signals and cytokines to sustain plasma cells for long periods, perhaps for several years. This may seem ludicrous in light of the present dogma that plasma cells only live for a few days or weeks at most, but the notion is not inconsistent with the large body of data showing prolonged antibody production after immunization (*68*, *70–72*). In this context, it is worth noting that in the case of inert antigens, plasma cells lodge in the bone marrow only after a booster injection (*70*) but are found in the bone marrow after just a single exposure to live virus (*73*). This suggests a relation between long-term antibody production and the presence of plasma cells in the bone marrow.

In contrast to prolonged serum antibody responses, the effector phase of the T cell response is short-lived. This dichotomy in the humoral and cellular responses is a feature of most acute viral infections (also of immunization, in general) and gave rise to the misconception that T cell "memory" is short-lived (*74*). Of course, numerous studies have now shown that the memory T cell response is indeed long-lived (*5*); it is the effector phase that is short-lived and seen only in the presence of antigen. This makes teleological sense. Sustained secretion or overproduction of cytokines can have deleterious effects on the immune system, and the presence of fully active killers could result in immunopathological damage if some of these CTLs were cross-reactive with self antigens. Thus, maintaining T cell immunity by sustaining the effector phase carries a high price tag. Because memory T cells can rapidly develop into effectors and quickly gain access to sites of infection, it is not essential, in most instances, to have preexisting effector T cells to provide protection. An exception to this might be certain mucosal infections in which clinical symptoms appear so quickly that there is not sufficient time for memory T cells to come into play. By the time memory T cells expand, differentiate into effectors, and control the infection, part or most of the damage is already done. In such situations, effector T cells at mucosal sites may be required to prevent disease. This may be the case in experimental models of RSV and rotavirus infections, in which it was reported that protective CTL immunity is short-lived (*75*). Because effector CTLs are strictly dependent on antigen, it is not surprising that this response waned rapidly as amounts of antigen declined.

Memory B and T cells do not prevent infection per se but they quickly proliferate and differentiate into effectors upon reexposure to pathogen; this rapid recall response is critical in controlling the extent of infection and preventing disease (*5*). Because both T and B cell memory are long-lived, memory responses are an important component of long-term protective immunity (*5*). However, memory responses in general are more effective in preventing disease due to systemic infection than to mucosal infection. In viruses such as measles and polio, virus replication at the site of entry (the respiratory tract for measles and the intestinal tract for polio) does not produce any clinical symptoms; disease results from viral spread to other tissues (*72*). In such systemic infections, there is enough time for memory T and B cells to expand, differentiate, control the infection, and prevent clinical disease. The window of opportunity is much shorter for pathogens that produce disease by replicating and causing tissue damage at the site of entry (for example, rotavirus in the gut and RSV in the respiratory tract) (*72*). In such mucosal infections, memory cells by themselves are unable to fully prevent clinical symptoms although recurrent infections tend to be less severe (*72*). Thus, in mucosal infections, immunological memory can remain intact, but protective immunity starts waning with the decline of effector cells at mucosal sites.

Conclusion

All four cell types—T and B effector cells and T and B memory cells—contribute to protective immunity against infectious diseases, but the relative importance of each cell type varies according to the nature of the pathogen and the type of disease it produces. Moreover, the four cell types exhibit different requirements for their maintenance. Identifying the correlates of immunity for different pathogens and understanding how these responses can be sustained is critical for the rational development of vaccines that will induce long-term protective immunity.

REFERENCES AND NOTES

1. P. L. Panum, *Virchows Arch.* 1, 492 (1847) [reprinted in *Med. Classics* 3, 829 (1939)].
2. J. H. Finley Jr., *The Complete Writings of Thucydides: The Peloponnesian War* (Modern Library, New York, 1951).
3. W. A. Sawyer, *J. Prev. Med.* 5, 413 (1931).
4. J. R. Paul, J. T. Riordan, J. L. Melnick, *Am. J. Hyg.* 54, 275 (1951).
5. P. C. Beverley, *Curr. Top. Microbiol. Immunol.* 159, 111 (1990); S. L. Swain *et al., Immunol. Rev.* 123, 115 (1991); E. Vitetta *et al., Annu. Rev. Immunol.* 9, 193 (1991); C. R. Mackay, *Adv. Immunol.* 43, 217 (1993); D. Gray, *Annu. Rev. Immunol.* 11, 49 (1993); J. Sprent, *Cell* 76, 315 (1994); R. Ahmed, *Semin. Virol.* 5, 319 (1994); J. Sprent and D. F. Tough, *Science* 265, 1395 (1994).
6. S. Oehen, H. Waldner, T. M. Kundig, H. Hengartner, R. M. Zinkernagel, *J. Exp. Med.* 176, 1273 (1992).
7. E. S. Razvi and R. M. Welsh, *Adv. Virus Res.* 45, 1 (1995).
8. L. L. Lau, B. D. Jamieson, T. Somasundaram, R. Ahmed, *Nature* 369, 648 (1994).
9. S. Hou, L. Hyland, K. W. Ryan, A. Portner, P. C. Doherty, *ibid.*, p. 652.
10. P. C. Doherty *et al., Annu. Rev. Immunol.* 10, 123 (1992).
11. R. A. Tripp, S. Hou, A. McMickle, J. Houston, P. C. Doherty, *J. Immunol.* 154, 5013 (1995); R. A. Tripp, J. M. Lahtl, P. C. Doherty, *ibid.* 155, 3719 (1995).
12. D. Moskophidis, U. Assmann-Wischer, M. M. Simon, F. Lehmann-Grube, *Eur. J. Immunol.* 17, 937 (1987); A. Gessner, D. Moskophidis, F. Lehmann-Grube, *J. Immunol.* 142, 1293 (1989).
13. T. Ueharа *et al., Blood* 80, 452 (1992); J. A. Levy, *AIDS* 7, 1401 (1993); G. Pantaleo, C. Graziosi, A. S. Fauci, *N. Engl. J. Med.* 328, 327 (1993); G. Pantaleo *et al., ibid.* 332, 209 (1995); Y. Cao *et al., ibid.*, p.228.
14. E. R. Kearney, K. A. Papa, D. Y. Loh, M. K. Jenkins, *Immunity* 1, 327 (1994).
15. A. Kelso *et al., Immunol. Rev.* 123, 85 (1991); S. L. Swain and L. M. Bradley, *Semin. Immunol.* 4, 59 (1992).
16. M. G. McHeyzer-Williams and M. M. Davis, *Science* 268, 106 (1995).
17. Y. Liu and C. A. Janeway, *J. Exp. Med.* 172, 1735 (1990); S. Webb, C. Morris, J. Sprent, *Cell* 63, 1249 (1990); Y. Kawabe and A. Ochi, *Nature* 349, 245 (1991); J. H. Russell *et al., Proc. Natl. Acad. Sci. U.S.A.* 88, 2151 (1991); T. Takahashi *et al., Cell* 76, 969 (1994); G. G. Singer and A. K. Abbas, *Immunity* 1, 365 (1994); F. Ramsdell *et al., Int. Innumol.* 6, 1545 (1994); S. Nagata and P. Golstein, *Science* 267, 1449 (1995); L. L. Carter and R. W. Dutton, *J. Immunol.* 155, 1028 (1995); M. Galvan and R. Ahmed, in preparation.
18. L. Zheng *et al., Nature* 377, 348 (1995).
19. U. Wurzburg, H. Schutt-Gerowitt, K. Rajewsky, *Eur. J. Immunol.* 3, 762 (1973); Y. Chien *et al., Nature* 309, 322 (1984); R. K. Strong *et al., Nature* 318, 577 (1985); M. Kronenberg, G. Siu, L. Hood, N. Shastri, *Annu. Rev. Immunol.* 4, 529 (1986); P. J. Fink, L. A. Matis, J. L. McElligott, M. Bookman, S. M. Hedrick, *Nature* 321, 219 (1986).
20. B. Zheng, W. Xue, G. Kelsoe, *Ibid.* 372, 556 (1994).
21. J. L. Maryanski *et al., Immunity* 4, 47 (1996).
22. M. Volkert, O. Marker, K. Bro-Jorgenson, *J. Exp. Med.* 139, 1329 (1974); E. D. Johnson and G. A. Cole, *Ibid.* 141, 866 (1975).
23. J.-C. Cerottini *et al., Ibid.* 140, 703 (1974); J.-C. Cerottini and H. R. MacDonald, *Annu. Rev. Immunol.* 7, 77 (1989).
24. S. L. Swain, *Immunity* 1, 543 (1994).
25. L. Bruno, J. Kirberg, H. von Boehmer, *Ibid.* 2, 37 (1995).
26. G. Berke, *Annu. Rev. Immunol.* 12, 735 (1994); D. Kägi *et al., Science* 265, 528 (1994); C. M. Walsh *et al., Proc. Natl. Acad. Sci. U.S.A.* 91, 10854 (1994).
27. D. F. Tough and J. Sprent, *Stem Cells* 13, 242 (1995); *J. Exp. Med.* 179, 1127 (1994).
28. S. J. Korsmeyer, *Blood* 80, 879 (1992); M. C. Raff, *Nature* 356, 397 (1992); S. Cory, *Curr. Opin. Immunol.* 13, 513 (1995); A. N. Akbar, M. Salmon, J. Savill, G. Janossy, *Immunol. Today* 14, 526 (1993); M. Salmon *et al., Eur. J. Immunol.* 24, 892 (1994).
29. T. Renno, M. Hahne, H. R. MacDonald, *J. Exp. Med.* 181, 2283 (1995).
30. J. Hotchin, *Monogr. Virol.* 3, (1971); R. Ahmed *et al., J. Exp. Med.* 60, 521 (1984); B. Rochs and H. von Boehmer, *Science* 251, 1225 (1991); D. Moskophi-

dis, F. Lechner, H. Pircher, R. M. Zinkernagel, *Nature* 362, 758 (1993); J. Kirberg, L. Bruno, H. von Boehmer, *Eur. J. Immunol.* 23, 1963 (1993); M. Metlioubian, R. J. Concepcion, R. Ahmed, *J. Virol.* 68, 8056 (1994); M. Battegay *et al.*, *ibid.*, p. 4700.
31. I. C. M. MacLennan, *Annu. Rev. Immunol.* 12, 117 (1994).
32. F. Celada, *Prog. Allergy* 15, 223 (1971); G. J. V. Nossal and G. L. Ada, *Antigens, Lymphoid Cells and the Immune Response* (Academic Press, New York, 1971); T. L. Feldbush, *Cell. Immunol.* 8, 435 (1973); T. E. Mandel, R. P. Phipps, A. Abbot, J. G. Tew, *Immunol. Rev.* 53, 29 (1980).
33. D. Kitamura, J. Roes, R. Kuhn, K. Rajewsky, *Nature* 350, 423 (1991).
34. R. N. Germain, *Cell* 76, 287 (1994); M. J. Bevan, *J. Exp. Med.* 182, 639 (1995); K. L. Rock, *Immunol. Today*, 17, 131 (1996).
35. D. Gray and P. Matzinger, *J. Exp. Med.* 174, 969 (1991).
36. A. Moltbacher, *Ibid.* 179, 317 (1994).
37. F. DiRosa and P. Matzinger, *Ibid.*, in press.
38. M. Asano and R. Ahmed, *Ibid.*, in press.
39. W. E. Demkowicz, R. A. Lattaua, J. Wang , F. A. Ennis, *J. Virol.*, in press.
40. A. Livingstone, in preparation.
41. D. Gray, M. H. Kosco, B. Stockinger, *Int. Immunol.* 3, 141 (1991).
42. P. Dullforce, D. van Essen, D. Gray, in preparation; M. Asano and R. Ahmed, in preparation.
43. P. Doherty, personal communication.
44. C. R. Mackay, W. L. Marston, L. Dudler, *J. Exp. Med.* 171, 801 (1990).
45. C. A. Michie, A. MacLean, C. Alcock, P. C. L. Beverley, *Nature* 360, 264 (1992).
46. E. S. Razvi, R. M. Welsh, H. I. McFarland, *J. Immunol.* 154, 620 (1995); L. L. Lau and R. Ahmed, in preparation.
47. J. F. A. P. Miller and G. F. Mitchell, *Transplant. Rev.* 1, 3 (1968).
48. P. C. L. Beverley, *Immunol. Today* 11, 203 (1990); H. von Boehmer and K. Hafen, *J. Exp. Med.* 177, 891 (1993); L. K. Selin, S. R. Nahill, R. M. Welsh, *Ibid.* 179, 1933 (1994); P. Matzinger, *Nature* 369, 605 (1994).
49. P. M. Allen, *Cell* 76, 593 (1994); S. C. Jameson, K. A. Hogquist, M. J. Bevan, *Annu. Rev. Immunol.* 13, 93 (1995).
50. D. Unutmaz, P. Pileri, S. Abrignani, *J. Exp. Med.* 180, 1159 (1994); J. Sprent, personal communication.
51. E. S. Bell, Sm. M. Sparshott, M. T. Drayson, W. L. Ford, *J. Immunol.* 139, 1379 (1987); B. Rocha, N. Deutigny, P. Pereira, *Eur. J. Immunol.* 19, 905 (1989).
52. G. Freer *et al.*, *Ibid.* 25, 1410 (1995).
53. C. Berek, G. M. Griffiths, C. Milstein, *Nature* 316, 412 (1984); C. Berek and C. Milstein, *Immunol. Rev.* 105, 5 (1988).
54. G. G. B. Klaus and J. H. Humphrey, *Immunology* 33, 31 (1977).
55. P.-J. Linton, D. Decker, N. R. Klinman, *Cell* 59, 1049 (1989); P.-J. Linton, D. Lo, L. Lai, G. J. Thorbecke, N. R. Klinman, *Eur. J. Immunol.* 22, 1293 (1992); P.-J. Linton and N. R. Klinman, *Semin. Immunol.* 4, 3 (1992).
56. J. Jacob and G. Kelsoe, *J. Exp. Med.* 176, 679 (1992).
57. Y.-J. Liu *et al.*, *Nature* 342, 929 (1989); D. Gray, P. Dullforce, S. Jainandunsing, *J. Exp. Med.* 180, 141 (1994); T. M. Foy *et al.*, *Ibid.*, p. 157.
58. M. F. Neurath, E. E. Max, W. Strober, *Proc. Natl. Acad. Sci. U.S.A.* 92, 5336 (1995).
59. E. Stuber, M. Neurath, D. Calderhead, H. F. Fell, W. Strober, *Immunity* 2, 507 (1995).
60. Y.-J. Liu *et al.*, *Eur. J. Immunol.* 21, 1107 (1991).
61. C. J. Turner, D. H. Mack, M. M. Davis, *Cell* 77, 297 (1994).
62. G. Kraal, I. L. Weissman, E. C. Butcher, *Nature* 298, 377 (1982); Y. J. Liu *et al.*, *Immunity* 2, 239 (1995).
63. J. Jacob, J. Przylepa, C. Miller, G. Kelsoe, *J. Exp. Med.* 178, 1293 (1993).
64. D. Gray and H. Skarvall, *Nature* 336, 70 (1988).
65. B. Schittek and K. Rajewsky, *Ibid.* 346, 749 (1991).
66. P. Vieira and K. Rajewsky, *Int. Immunol.* 2, 487 (1990).
67. M. H. Kosco-Vilbois and D. Gray, unpublished observation.
68. I. Scheibel *et al.*, *Acta Pathol. Microbiol. Scand.* 67, 380 (1966); D. Simonsen, K. Kjeldsen, I. Heron, *Lancet* II, 1240 (1964); K. Kjeldsen, O. Simonsen, I. Heron, *Ibid.* I, 900 (1965); D. Cohen *et al.*, *Eur. J. Epidemiol.* 10, 267 (1994).
69. F. Ho, J. E. Lortan, I. C. M. MacLennan, M. Khan, *Eur. J. Immunol.* 16, 1297 (1986).
70. R. Banner, W. Hijmans, J. J. Haaijman, *Clin. Exp. Immunol.* 46, 1 (1981).
71. J. J. Miller, *J. Immunol.* 92, 673 (1964).
72. S. A. Plotkin and E. A. Mortimer, Eds., *Vaccines* (Saunders, Philadelphia, PA, ed. 2, 1994); B. N. Fields, *Fields Virology* (Lippincott-Raven, Philadelphia, PA, 1996).
73. L. Hyland, M. Sangster, R. Sealy, C. Coleclough, *J. Virol.* 68, 6083 (1994); M. F. Bachmann *et al.*, *J. Immunol.* 153, 3388 (1994); M. K. Slifka, M. Matloubian, R. Ahmed, *J. Virol.* 69, 1895 (1995).
74. R. M. Zinkernagel, *Curr. Top. Microbiol. Immunol.* 159, 65 (1990).
75. A. B. Kulkarni *et al.*, *J. Virol.* 67, 1044 (1993); M. A. Franco and H. R. Greenberg, *ibid.* 69, 7800 (1995).
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Lymphocyte Homing and Homeostasis

Eugene C. Butcher and Louis J. Picker

The integration and control of systemic immune responses depends on the regulated trafficking of lymphocytes. This lymphocyte "homing" process disperses the immunologic repertoire, directs lymphocyte subsets to the specialized microenvironments that control their differentiation and regulate their survival, and targets immune effector cells to sites of antigenic or microbial invasion. Recent advances reveal that the exquisite specificity of lymphocyte homing is determined by combinatorial "decision processes" involving multistep sequential engagement of adhesion and signaling receptors. These homing-related interactions are seamlessly integrated into the overall interaction of the lymphocyte with its environment and participate directly in the control of lymphocyte function, life-span, and population dynamics. In this article a review of the molecular basis of lymphocyte homing is presented, and mechanisms by which homing physiology regulates the homeostasis of immunologic resources are proposed.

The immune system faces daunting challenges in its mission of protecting the body from microbial invasion. From a large but finite number of antigen-receptor–defined lymphocyte clones, it must establish and maintain a diverse, nonautoimmune population of mature lymphocytes and endow them with the capability to respond to foreign antigen wherever it may enter the body. It must control the interplay between B cells, T cells, specialized accessory cell populations, and antigen so as to efficiently initiate primary cellular and humoral immune responses. It must integrate immune responses throughout the body, while at the same time targeting and permitting specialization of immune response modalities in different regions of the body such as the alimentary tract, the lung, and the skin. Finally, the immune system must use efficient mechanisms of homeostasis to provide for long-lasting but malleable immunity over time and to prevent the overexpansion or depletion of specialized lymphocyte subsets. To accomplish these diverse tasks, evolution has created a dispersed system of highly specialized immune microenvironments that control the differentiation and homeostasis of mature lymphocytes and then linked these microenvironments together with each other and with the effector sites of the body through an elaborate system of lymphocyte homing and recirculation.

Our purpose in this review is to describe recent molecular and conceptual advances in our understanding of lymphocyte recirculation and homing; to emphasize the importance of targeted lymphocyte migration in the integration, regulation, and specialization of immune responses; and to explore emerging concepts of the role of recirculation and microenvironmental homing in immune homeostasis.

Lymphocyte Recirculation and Homing from the Blood

Most mature lymphocytes recirculate continuously, going from blood to tissue and back to blood again as often as one to two times per day (*1*). Recirculation is not random, but rather is targeted by active mechanisms of lymphocyte–endothelial cell rec-

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